



The threat of drug-resistant malaria – trends and applicability of molecular markers of drug resistance

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Malaria control in Sub-Saharan Africa (SSA)

Success in control of *P. falciparum* in SSA mainly due to:

Implementation of interventions/measures:

- Preventive measures (ITNs, Indoor residual spraying (IRS))
- Improved diagnostics (RDTs)

Efficacious drugs to treat malaria:

- *Artemisinin-based combination Therapies (ACTs)*
 - No drug resistance in SSA

Preventive drug treatment:

Intermittent preventive treatment of infants (IPTi),

IPT of pregnant women (IPTp)

Children (seasonal malaria chemoprevention (SMC))

**Major threat against the control of malaria in SSA:
drug resistance**

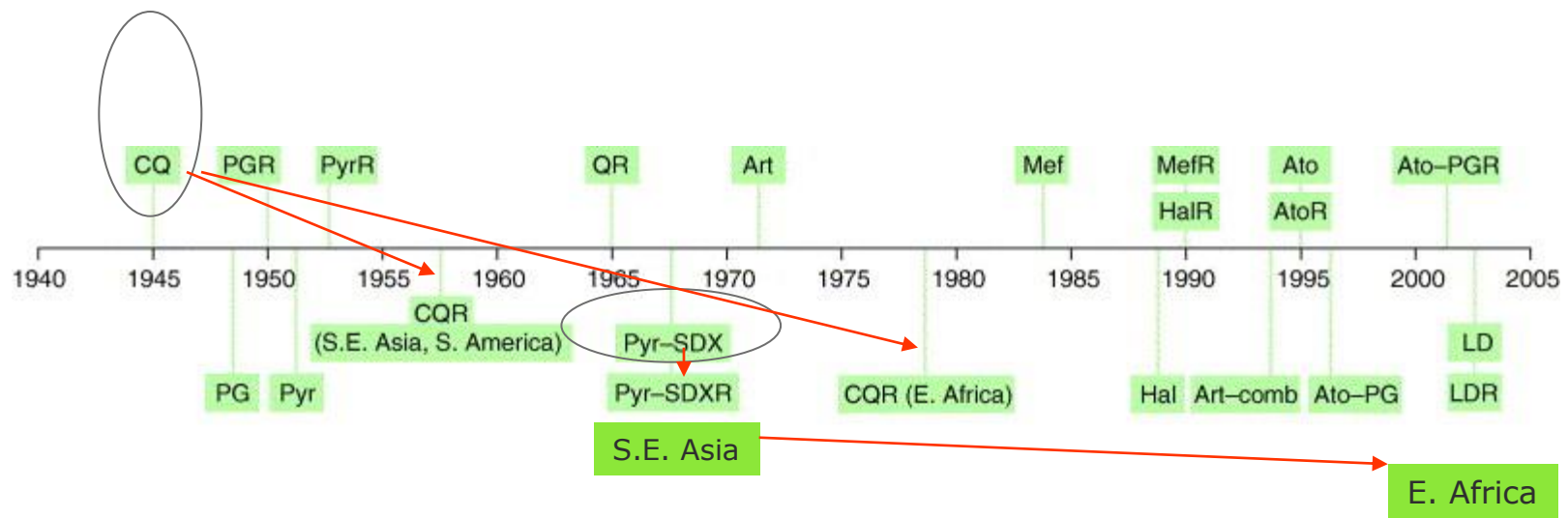


Malaria control – the challenge of drug resistance

Learning from history; resistance will always emerge

Chloroquine

Pyrimethamine/sulfadoxine (Fansidar®) - SP



Hyde. Trends in Parasitol. Vol.21 No.11 (2005)

A continuous need to monitor drug resistance

Malaria control – Identifying resistance

- *In vivo* drug efficacy trials and *In vitro* drug susceptibility tests
- Molecular makers of drug resistance: SNPs in *Pf* genes => resistance to antimalarial drugs

Drug	Resistance gene	Main SNPs (aa at position)		NB
Chloroquine	<i>Pfcrt</i>	72-76 CVMNK (WT) 72-76 CVIET (MT) 72-76 SVMNT (MT)		
Pyrimethamine	<i>Pfdhfr</i>	N51I C59R S108N	51+59+108 => 3 MT	High P resistance
Sulfadoxine	<i>Pfdhps</i>	A437G K540E	437+540=> 2 MT (+ <i>Pfdhfr</i> => 5MT)	High SP resistance
		A581G	+581=> 3 MT (+ <i>Pfdhfr</i> => 6MT)	Super SP resistance

Evolution of SP resistance: 108+51/59 MT in *Pfdhfr*
then 437, 437+540, 437+540+581 MT in *Pfdhps*

Extremely Trump-like genes



Molecular markers of drug resistance to guide policy - IPTi

If we consecutively can map markers of drug resistance =>

Surveillance of drug resistance:

- Change in prevalence of mutations causing resistance over time =>
- Change in drug policy

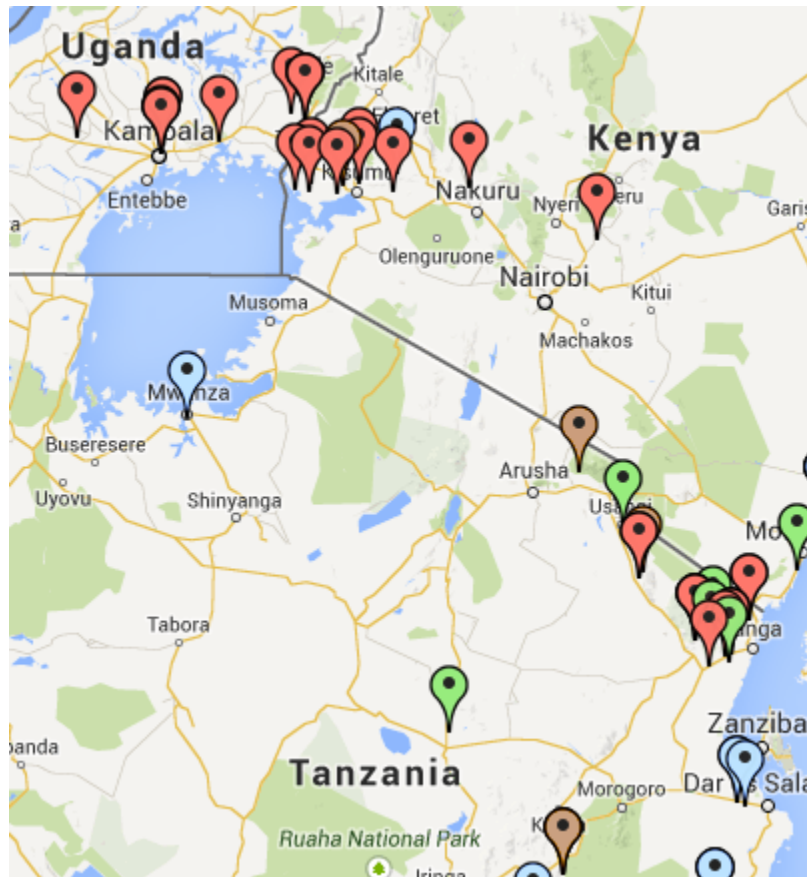
(if you have a drug to change to...)

- Examples: SP (IPTi and IPTp) and CQ



The use of molecular markers of drug resistance

- Often molecular markers have merely confirmed that there is resistance *in vivo*:



- Prevalence of 540E (high SP resistance) confirms that there is high SP resistance *in vivo* in East Africa

Percentage of data set
found to exhibit mutation



(Sampled at various time points
(low prev. = earlier studies))

Molecular markers of drug resistance to guide policy - IPTi

SP is still recommended for IPTi (as policy: only Burkina Faso)

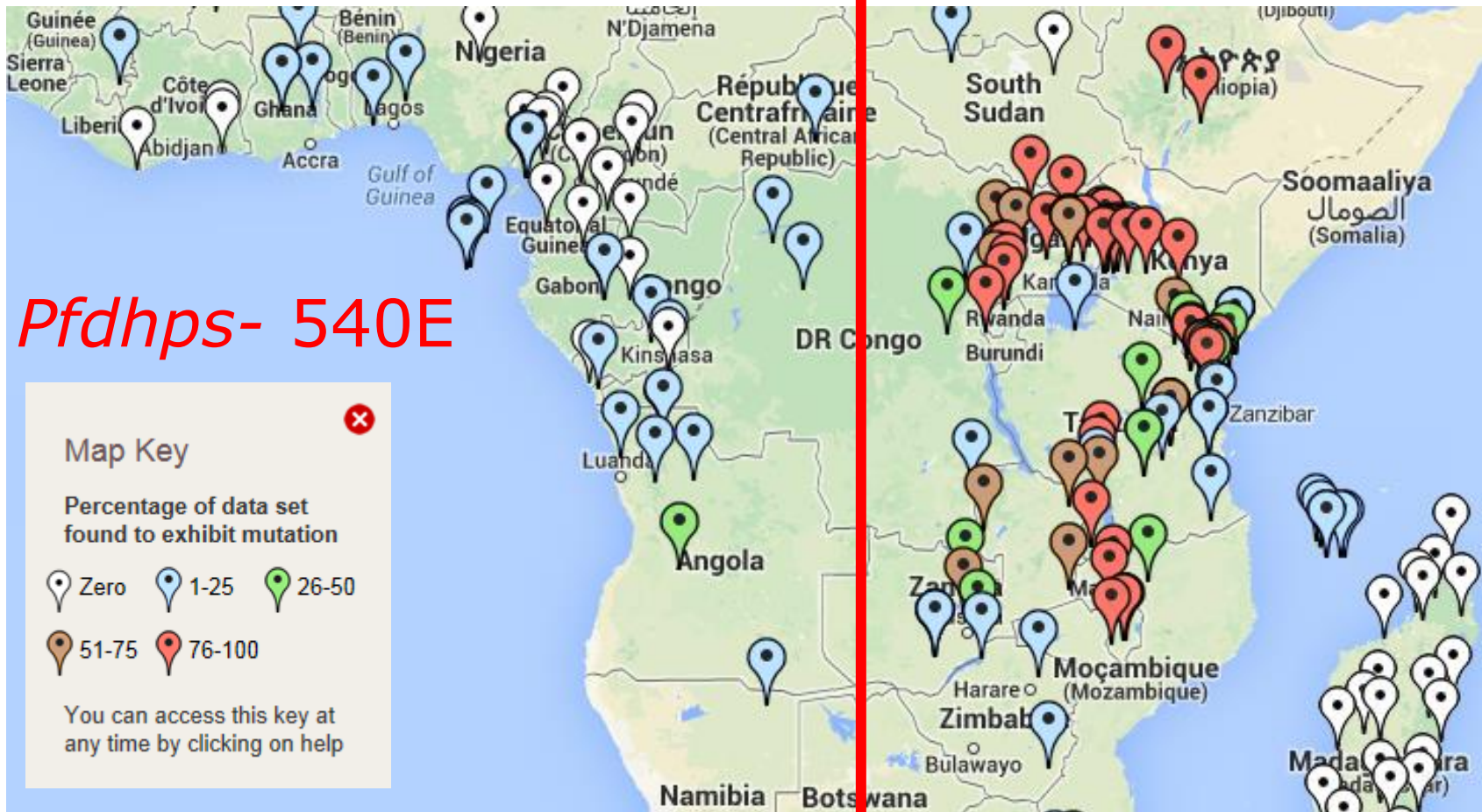
- IPTi: provide effective protection against multiple infections and etc. *Aponte et al. Lancet, Vol 374, 2009*
- However, in countries such as Tanzania with high level of SP resistance, no protective effect *Gosling et al. Lancet Vol 374, 2009*
 - As a result of high prevalence of 5MT in *Pfdhfr* and *Pfdhps* (measured as high prevalence of 540 MT)
- Threshold for when to use IPTi; based on molecular data:
 - WHO recommend only to use SP for IPTi

"...where parasite resistance to SP is not high – defined as a prevalence of the Pfdhps 540 mutation of $\leq 50\%$."

http://www.who.int/malaria/news/WHO_policy_recommendation_IPTi_032010.pdf?ua=1



Thresholds for protective efficacy of IPTi



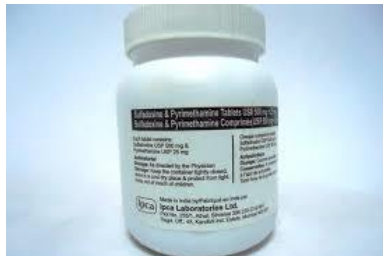
Molecular markers of drug resistance to guide policy - IPTp

SP is used as IPT for pregnant women (IPTp)

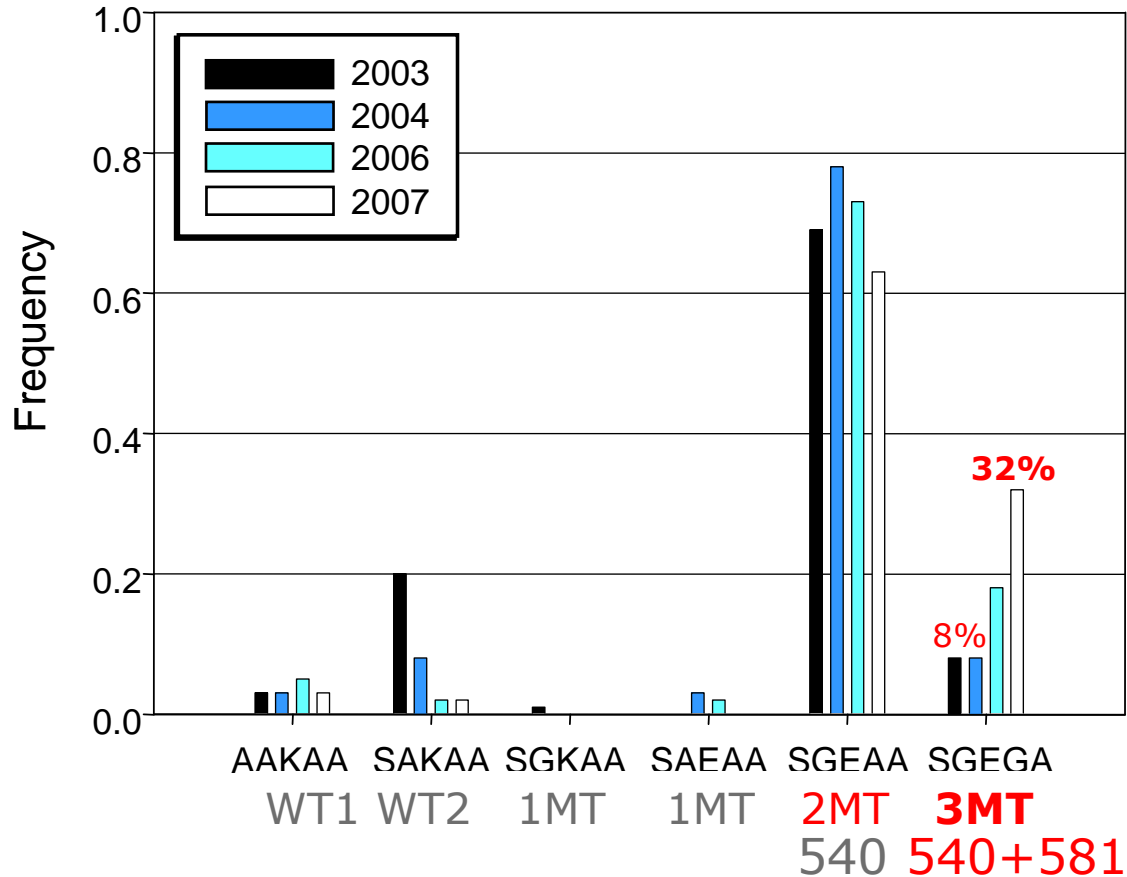
- IPTp-SP for all pregnant women at each scheduled antenatal care visit until the time of delivery (3 times, one month apart)
- IPTp-SP prevents: placental infection, clinical malaria, maternal and fetal anaemia, low birth weight and neonatal mortality

Eisele et al. *Lancet Infect Dis.* 2012 Dec;12(12):942-9.

- IPTp seems effective even in areas with high prev. of 540 MT;
however, what if an additional mutation emerged?



Prevalence of *Pfdhps* haplotypes in Korogwe, Tanzania –



Alifrangis et al. (2009), *Am J Trop Med Hyg*, 80(4), 2009

Is IPTp still effective in areas where we now have the additional MT? (now; 6MT in *Pfdhfr* and *Pfdhps*)



The use of molecular markers of drug resistance - IPTp

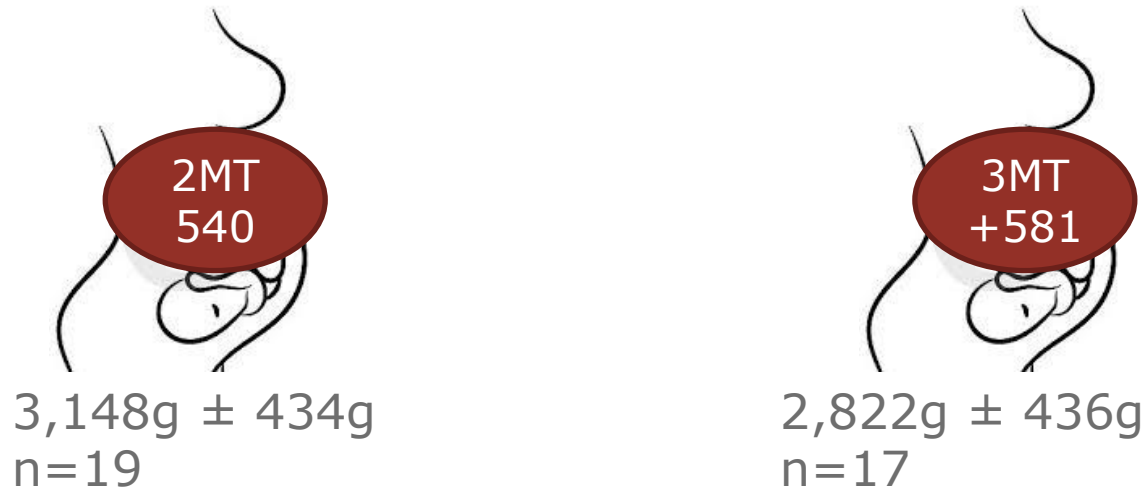
STOPPAM – Strategies to Prevent Pregnancy Associated Malaria

- Cohort of pregnant women living in Korogwe, NE-Tanzania
- Sep. 2008 – Oct. 2010
- 995 women included, all on IPTp
- Low prevalence of malaria; Among the women completing follow-up, 76 had a total of 96 episodes of malaria.
- Prevalence of 540 MT = 100%, **581 MT = 54%**

Minja et al. *Emerg Infect Dis.* 2013;19(9).



Molecular markers of drug resistance to guide policy - IPT



2MT vs. 3MT infections =>
mean reduction of birthweight of 326g

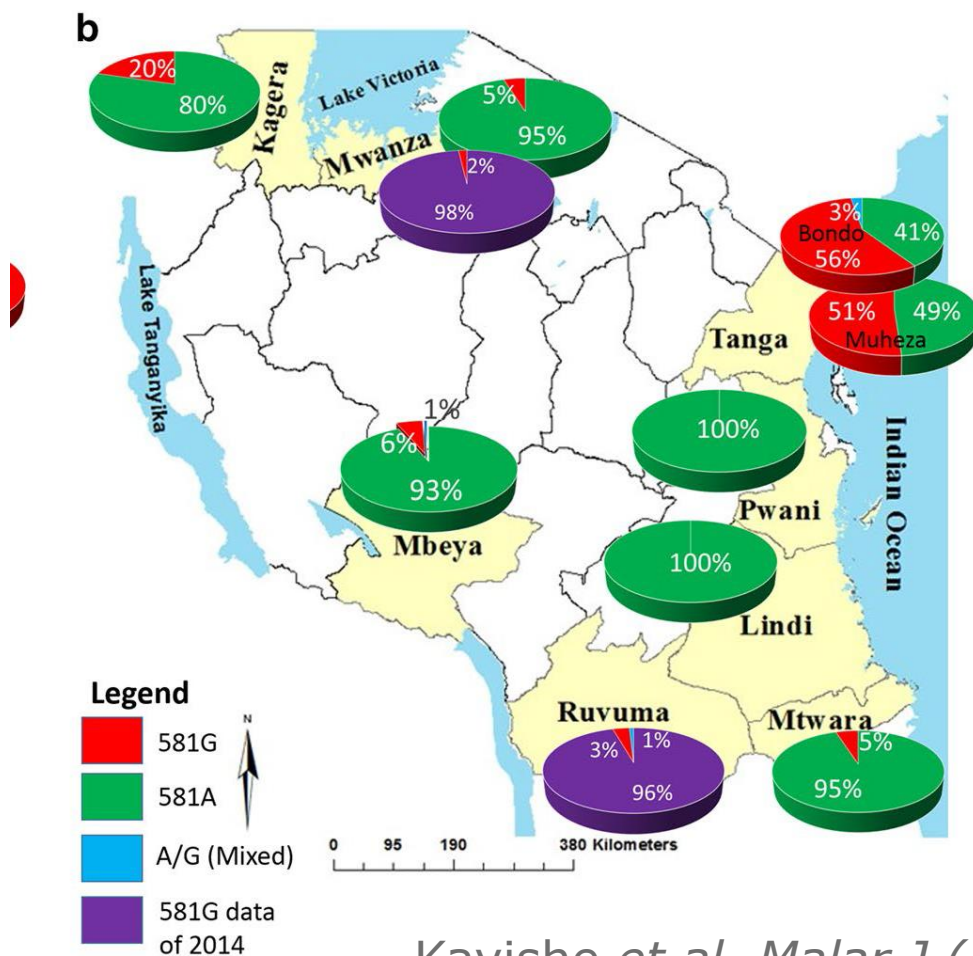
Minja et al. *Emerg Infect Dis.* 2013;19(9).

"Consider discontinuing IPTp-SP when the population prevalence of...mutation 540E is greater than 95%, AND the prevalence of mutation 581G is greater than 10%, as it is likely to be ineffective"

http://www.who.int/malaria/mpac/mpac_sep13_erg_ipt_malaria_pregnancy_report.pdf



Molecular markers of drug resistance to guide policy - IPT



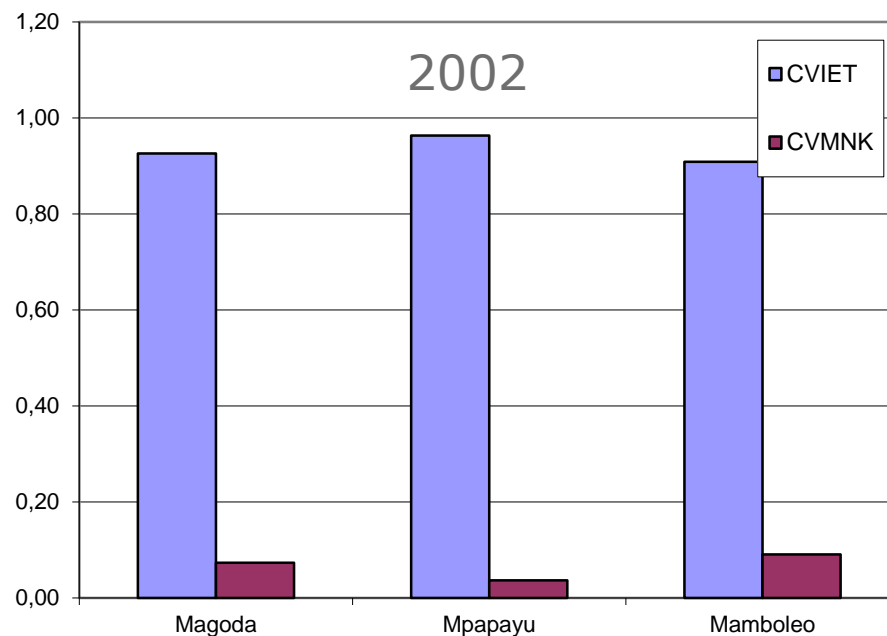
Kavishe et al. *Malar J* (2016) 15:335



Molecular markers of drug resistance to guide policy - CQ

Can molecular markers be used to measure increased sensitivity of *P. falciparum* to abandoned drugs, e.g. Chloroquine (CQ)?

Ex. In Tanzania; CQ was abandoned in 2001 (officially)



- Tanga, NE-Tanzania
- >95% prev. Of CQ resistant parasites

- Now..

(Unpublished data)



Molecular markers of drug resistance to guide policy - CQ

Region	Prev. Of CVMNK Wildtype (%)
Tanga	93.2
Coastal	93.5
Mtwara	93.2
Kagara	85.7
Mwanza	88.4
Mbeya	92.7
Overall	91.0

Across Tanzania: >85% of parasites are wildtypes and thus, susceptible to CQ

Mohammed et al., 2013 Malaria J: 12:415

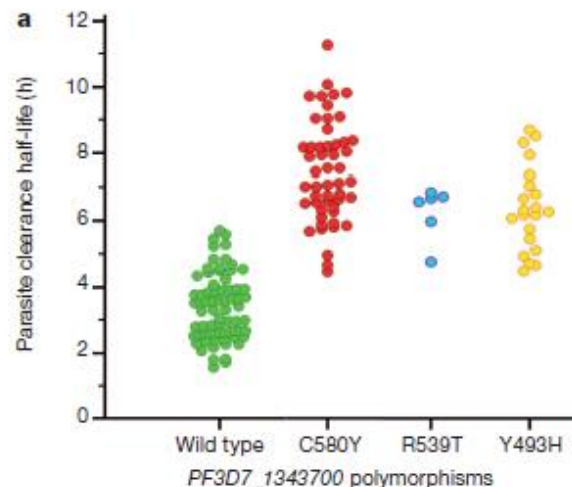


Molecular markers of drug resistance to guide policy - ACT

A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria

Frédéric Aréy^{1,2†}, Benoit Witkowski³, Chanaki Amaratunga⁴, Johann Beghain^{1,2†}, Anne-Claire Langlois^{1,2}, Nimol Khim³, Saorin Kim³, Valentine Duru³, Christiane Bouchier⁵, Laurence Ma⁵, Pharath Lim^{3,4,6}, Rithea Leang⁶, Socheat Duong⁶, Sokunthea Sreng⁶, Seila Suon⁶, Char Meng Chuor⁶, Denis Mey Bout⁷, Sandie Ménard^{8†}, William O. Rogers⁹, Blaise Genton¹⁰, Thierry Fandeur^{1,3}, Olivo Miotto^{11,12,13}, Pascal Ringwald¹⁴, Jacques Le Bras¹⁵, Antoine Berry^{8†}, Jean-Christophe Barale^{1,2†}, Rick M. Fairhurst^{4*}, Françoise Benoit-Vical^{16,17*}, Odile Mercereau-Puijalon^{1,2*} & Didier Ménard^{3*}

Resistance to artesunate => K13-propeller polymorphisms



- A marker of ACT resistance *before* ACT resistance has been reported in SSA
- However, we do not know which SNPs will be important in SSA



Molecular markers of drug resistance to guide policy - ACT

Drug	Resistance gene	Main SNPs (aa at position)		NB
Chloroquine	<i>Pfcr</i>	72-76 CVMNK (WT) 72-76 CVIET (MT) 72-76 SVMNT (MT)		
Pyrimethamine	<i>Pfdhfr</i>	N51I C59R S108N	51+59+108 => 3 MT	High P resistance
Sulfadoxine	<i>Pfdhps</i>	A437G K540E A581G	436+540 => 2 MT +581=> 3 MT	High SP resistance <i>Super</i> SP resistance
Various antimalarials	<i>Pfmdr1</i>	N86Y, Y184F, S1034C, 1048, D1246N		
Artemisinin	K13	Various		



Determining molecular markers of drug resistance

PCR followed by various methodologies for SNP detection

- RFLP (enzymes cut to distinguish either WT or MT)
- Allele specific PCR
- Sequence specific oligonucleotide probe – ELISA

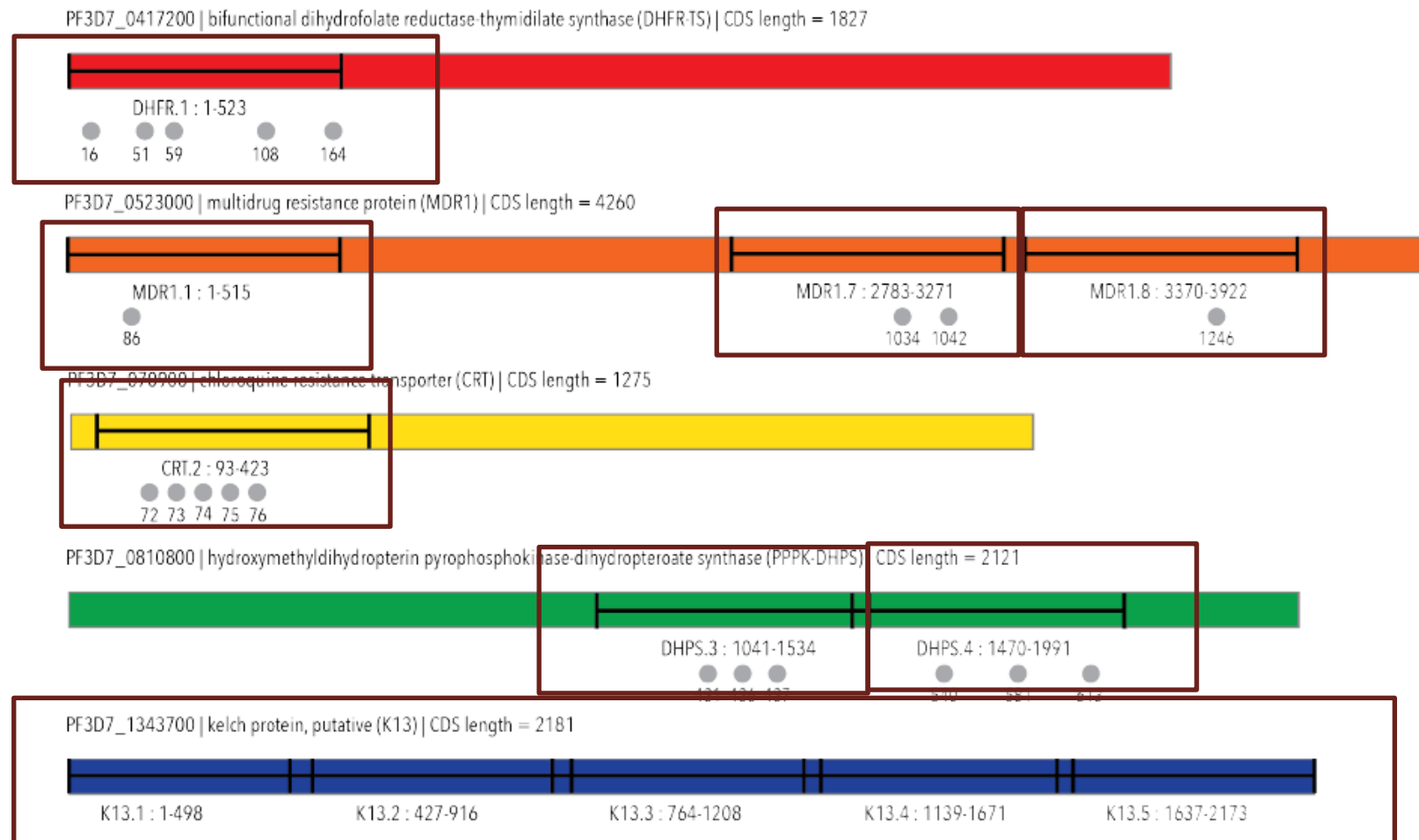
K13...sequencing is necessary:

- Sanger sequencing
- NGS...
 - (DTU Profs Ole Lund/Frank Aarestrup)



Determining molecular markers of drug resistance using NGS

Figure 3. Amplicon design



Sidsel Nag, unpublished

Determining molecular markers of drug resistance using NGS

1. Gene specific PCR:

PCR products of:

Multiplex 1

Mdr1 (1)
K13 (1)
K13(3)
K13(5)

Multiplex 2

Dhfr (1)
K13 (4)

Multiplex 3

Mdr (8)
Dhps (3)
K13 (3)

Simplex

Crt 2

DHFR.1 PF3D7_0417200

Fw. TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGATGGAACAAGTCTGCGACGTTTTCGA
Rev. GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAAAAATTCTTGATAAACAACGGAACCTCC

Non-annealing (not dhfr-related overhang)
(will be incorporated into PCR products)

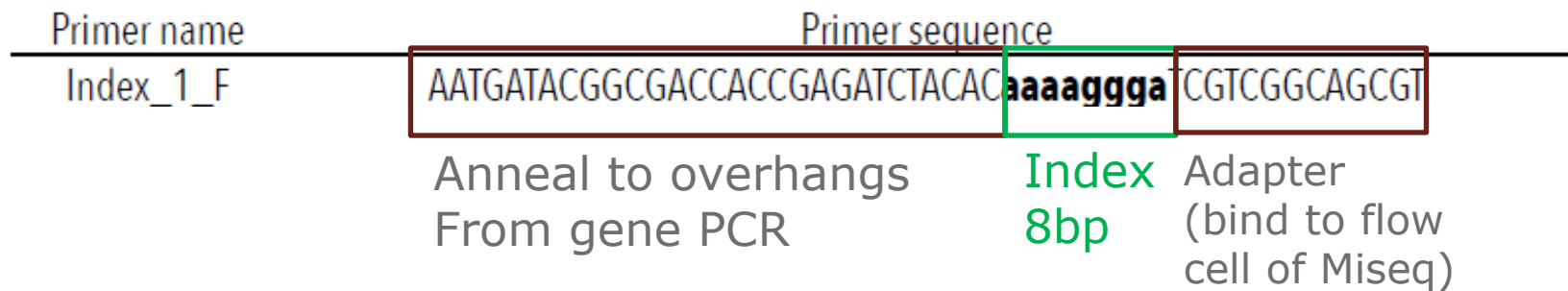
Gene Sp. Seq.

Sidsel Nag, unpublished



Determining molecular markers of drug resistance using NGS

2. Index PCR (necessary to link a Miseq sequence to a individual sample)



Indexing primers:

- Anneal to the overhangs
- Contain overhangs consisting of individual 8-base indices
- Adapter sequences that will allow the final PCR product to bind to the flow cell.

Indexing 8bp sequence:

50 variants in forward primers
50 variants in reverse primers
2450 combinations!

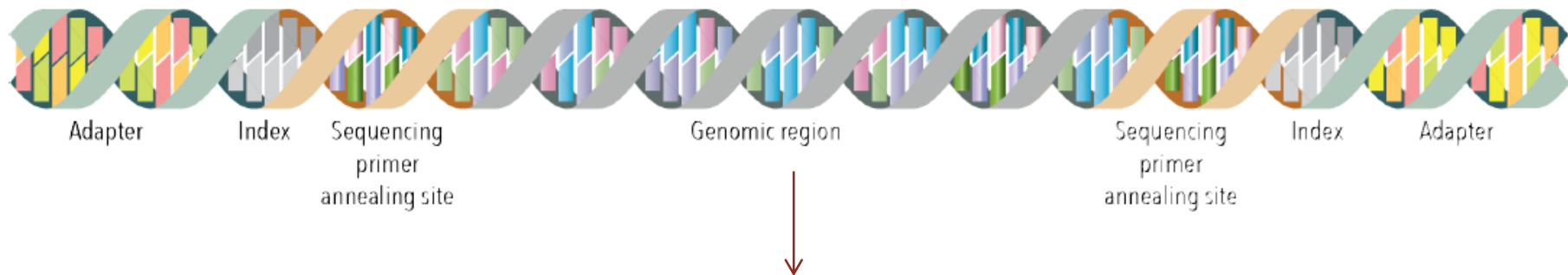
Sidsel Nag, unpublished



Determining molecular markers of drug resistance using NGS

Index PCR: using unique combinations of 8bp primers for each individual sample (consisting of fragments of all target genes)
Samples are...Indexed/barcoded

PCR product:



Pooling of Index PCRs

Miseq

Sidsel Nag, unpublished

Determining molecular markers of drug resistance using NGS

	A	C	D	E	F	G	BC	BD	BE	BF	BG	BH
#CHROM	POS	ID	REF - DHPS	ALT	QUAL	GB1-10C	GB1-10D	GB1-10E	GB1-10F	GB1-10G	GB1-10H	
4 PF3D7_0810800	1303	.	T	.	283:24:00	0/0:4530:0	0/0:4490:0	0/0:542:0	0/0:2651:0	0/0:5467:0	0/0:5172:0	
5 PF3D7_0810800	1304	.	C	.	283:24:00	0/0:4185:0	0/0:4212:0	0/0:542:0	0/0:2511:0	0/0:5123:0	0/0:4867:0	
6 PF3D7_0810800	1305	.	C	.	283:24:00	0/0:3895:0	0/0:3940:0	0/0:541:0	0/0:2377:0	0/0:4802:0	0/0:4212:0	
7 PF3D7_0810800	1306	.	T	G	222	0/0:4400:0	0/0:4457:0	0/0:542:0	1/1:2672:255	0/0:5347:0	0/0:4778:0	
8 PF3D7_0810800	1307	.	C	T	283:24:00	0/0:4213:0	0/0:4245:0	0/0:543:0	0/0:2536:0	0/0:5148:0	0/0:4869:0	
9 PF3D7_0810800	1308	.	T	.	283:24:00	0/0:4809:0	0/0:4789:0	0/0:543:0	0/0:2859:0	0/0:5829:0	0/0:4924:0	
10 PF3D7_0810800	1309	.	G	.	266.94	0/0:3922:0	0/0:4037:0	0/0:541:0	0/0:2292:0	0/0:4894:0	0/0:4907:0	
11 PF3D7_0810800	1310	.	G	C,T,A	222	1/1:3824	1/1:3908	1/1:540	1/1:2230	0/0:5518	1/1:4805	
12 PF3D7_0810800	1311	.	T	.	283:24:00	0/0:4172:0	0/0:4220:0	0/0:543:0	0/0:2507:0	0/0:5545:0	0/0:5042:0	
13 PF3D7_0810800	1312	.	C	.	283:24:00	0/0:3913:0	0/0:3991:0	0/0:541:0	0/0:2292:0	0/0:4924:0	0/0:4912:0	
14 PF3D7_0810800	1313	.	C	.	283:24:00	0/0:4297:0	0/0:4280:0	0/0:537:0	0/0:2535:0	0/0:5318:0	0/0:4787:0	
15 PF3D7_0810800	1314	.	T	.	262.98	0/0:3991:0	0/0:4071:0	0/0:542:0	0/0:2310:0	0/0:5055:0	0/0:4934:0	
16 PF3D7_0810800	1315	.	T	.	276:37:00	0/0:4008:0	0/0:4009:0	0/0:543:0	0/0:2320:0	0/0:4951:0	0/0:4891:0	
17 PF3D7_0810800	1316	.	T	.	283:24:00	0/0:4382:0	0/0:4339:0	0/0:543:0	0/0:2538:0	0/0:5411:0	0/0:5017:0	
18 PF3D7_0810800	1317	.	T	.	283:24:00	0/0:4586:0	0/0:4557:0	0/0:542:0	0/0:2725:0	0/0:5596:0	0/0:4880:0	
19 PF3D7_0810800	1318	.	G	.	283:24:00	0/0:4845:0	0/0:4724:0	0/0:541:0	0/0:2874:0	0/0:5819:0	0/0:5013:0	
20 PF3D7_0810800	1319	.	T	.	283:24:00	0/0:4850:0	0/0:4743:0	0/0:543:0	0/0:2851:0	0/0:5826:0	0/0:5084:0	

Sidsel Nag, unpublished



